Cell, Vol. 92, 265–277, January 23, 1998, Copyright ©1998 by Cell Press

# MIX-1: An Essential Component of the *C. elegans* Mitotic Machinery Executes X Chromosome Dosage Compensation

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## Summary

**We show that a functional component of the *C. elegans* mitotic machinery regulates X chromosome gene expression. This protein, MIX-1, is a member of the dosage compensation complex that associates specifically with hermaphrodite X chromosomes to reduce their gene expression during interphase. MIX-1 also associates with all mitotic chromosomes to ensure their proper segregation. Both dosage compensation and mitosis are severely disrupted by *mix-1* mutations. MIX-1 belongs to the SMC protein family required for mitotic chromosome condensation and segregation in yeast and frogs. Thus, an essential, conserved component of mitotic chromosomes has been recruited to the dosage compensation process. Rather than dosage compensation and mitosis being achieved by two separate sets of related genes, these two processes share an identical component, indicating a common mechanism for establishing higher order chromosome structure and proper X chromosome gene expression.**

## Introduction

Dosage compensation is an essential, chromosome-wide regulatory process that equalizes expression of X-linked genes between males (typically XO or XY) and females (typically XX), despite their 2-fold difference in X chromosome dose. Unique mechanisms of dosage compensation have evolved in flies, worms, and mammals, each arising as a consequence of the chromosome-based mechanism used to determine sex (reviewed in Cline and Meyer, 1996; Willard, 1996; Kuroda and Meller, 1997). Although the strategies used to achieve dosage compensation are diverse, all appear to involve global changes in X chromosome structure that ultimately serve to adjust the level of X-linked transcripts in only one sex. This adjustment in gene expression prevents the male- or female-specific lethality that would otherwise result from an incorrect level of X chromosome products.

In the nematode *Caenorhabditis elegans*, hermaphrodites (XX) halve the level of transcripts produced by each of their two X chromosomes to attain the same level produced by the single X of males (XO) (Meyer and Casson, 1986). This global repression of gene activity in hermaphrodites is superimposed upon the gene-specific spatial and temporal regulation of X-linked loci common to both sexes. Failure to activate dosage compensation in hermaphrodites causes overexpression of X-linked genes and lethality (Meyer and Casson, 1986; Nusbaum and Meyer, 1989; Plenefisch et al., 1989). Conversely, activation of dosage compensation in males causes an inappropriate reduction of X chromosome gene expression and lethality (Miller et al., 1988; Rhind et al., 1995).

Nematode dosage compensation is implemented by a large protein complex that localizes specifically to both X chromosomes of hermaphrodites around the 30-cell stage of embryogenesis (Chuang et al., 1994; Lieb et al., 1996). This biochemically defined complex includes DPY-26, DPY-27, and at least two additional proteins of ~150 and ~160 kDa (Chuang et al., 1996). Its localization to X depends on two hermaphrodite-specific genes, encoding SDC-2 and SDC-3 proteins, which coordinately control sex determination and dosage compensation (Chuang et al., 1996; Lieb et al., 1996; Davis and Meyer, 1997). The X localization of DPY-27 and the sequence similarities between DPY-27 and proteins involved in mitotic chromosome condensation led to speculation that this dosage compensation complex regulates gene expression by altering X chromosome structure (Chuang et al., 1994).

DPY-27 belongs to the evolutionarily conserved SMC (structural maintenance of chromosomes) family of proteins that participate in diverse chromosome behaviors including sister chromatid cohesion, mitotic chromosome condensation, and mitotic recombination repair (Chuang et al., 1994; Guacci et al., 1997; Hirano et al., 1997; Michaelis et al., 1997; reviewed in Koshland and Strunnikov, 1996). The SMC proteins comprise different subfamilies that can function together, probably as heterodimers. For example, two SMC homologs in *Xenopus laevis*, XCAP-C and XCAP-E, are components of the 13S condensin complex that drives mitotic chromosome condensation in vitro (Hirano et al., 1997). DPY-27 is distinct from other SMC family members in that it plays no role in mitosis or other general chromosome behaviors and associates only with X chromosomes (Chuang et al., 1994).

Inference that aspects of X chromosome structure might be involved in dosage compensation also stemmed from studies of DPY-26, which established a functional link between dosage compensation and meiotic chromosome segregation (Lieb et al., 1996). DPY-26 is required for the faithful segregation of chromosomes during meiosis. DPY-26 implements this role through its association with all meiotic chromosomes in germ cells, a cell type that may not undergo dosage compensation. Approximately 4% of meiotic chromosomes fail to disjoin and therefore segregate improperly in *dpy-26* mutants of both sexes. In contrast, virtually all *dpy-26* XX mutants die from the disruption of dosage compensation, suggesting that DPY-26 plays a more central role in dosage compensation than in meiosis. DPY-26 shares

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two small motifs with two mitotic proteins: XCAP-H, a component of the *X. laevis* 13S condensin complex (Hirano et al., 1997), and Barren, a *Drosophila* protein essential for mitotic chromosome segregation in vivo (Bhat et al., 1996). Despite the similarities, DPY-26 differs significantly from both proteins and does not function in mitosis.

The sequence similarities between conserved mitotic proteins and two key components of the dosage compensation complex, DPY-26 and DPY-27, raise a fundamental question about the relationship between dosage compensation and mitosis: Are these processes related simply because dosage compensation–specific proteins regulate X chromosome expression by exploiting the structural motifs used by mitotic proteins for chromosome segregation, or does the relationship have a deeper mechanistic significance, with conserved mitotic proteins actively participating in both chromosome segregation and dosage compensation within the same cell? The discovery of shared components would imply that the mechanism underlying chromosome condensation and transcriptional silencing in mitosis is directly related to the mechanism of dosage compensation. Hence, information about the mechanism of one process would be directly relevant to the mechanism of the other. Moreover, one could infer that the dosage compensation process evolved by recruiting existing components used in other chromosome behaviors to the new task of fine-tuning X chromosome expression. Thus, the evolution of dosage compensation would not have required the de novo creation of all its components.

In the present study, we describe a new component of the *C. elegans* dosage compensation complex that is essential to both sexes. We show that the ~150 kDa member of the dosage compensation complex, a protein we call MIX-1 (**mi**tosis and **X**-associated protein), is a homolog of Smc2p from yeast and XCAP-E from frogs. Not only is MIX-1 critical for repression of X chromosome gene expression in hermaphrodites, but it is also essential for mitotic chromosome segregation in both sexes. MIX-1 associates specifically with hermaphrodite X chromosomes during interphase and with all chromosomes of both sexes during mitosis. Thus, an evolutionarily conserved component of the mitotic machinery has been recruited to the dosage compensation process to regulate chromosome-wide gene expression. This functional link between the chromosome condensation required for mitosis and the fine tuning of X chromosome expression during interphase provided by MIX-1 implies a common mechanism for both processes. The dosage compensation function of MIX-1, but not its mitotic function, is dependent on DPY-27 and other regulators of dosage compensation. Therefore, a single SMC protein can participate in multiple protein complexes that mediate different chromosome behaviors in the same cell, with each activity being specified through interactions with other proteins. These combined observations suggest that dosage compensation arose in *C. elegans* through the evolution of dosage compensation–specific proteins that direct functional components of mitotic chromosomes to interphase X chromosomes.

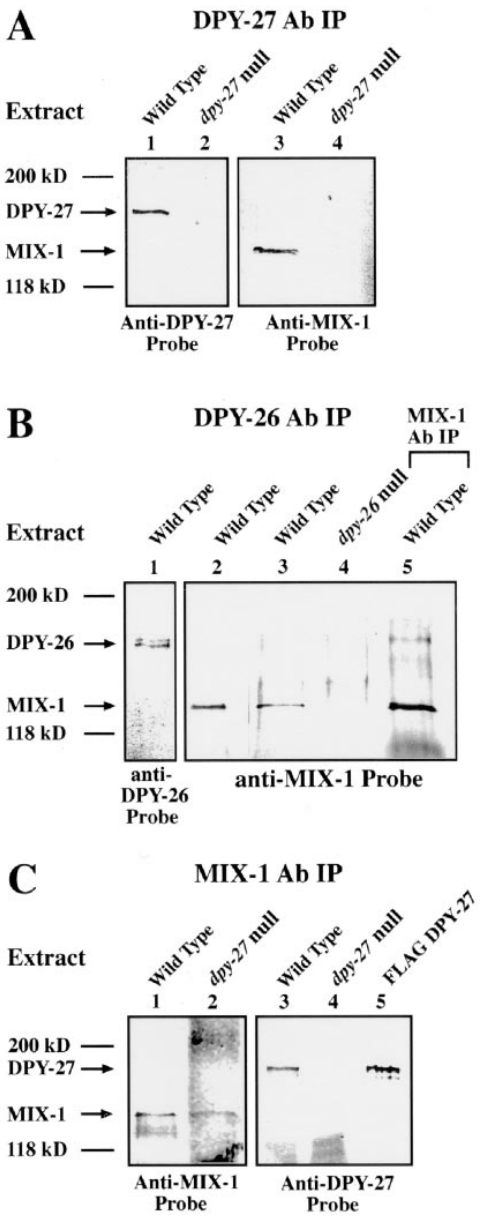


Figure 1. MIX-1 Is a Member of the Dosage Compensation Complex

(A) Western blots of immunoprecipitation reactions (IPs) using DPY-27 antibodies and nuclear extracts from wild-type or *dpy-27* mutant embryos that were probed with either DPY-27 or MIX-1 antibodies. DPY-27 antibodies immunoprecipitate DPY-27 and MIX-1 from wild-type (lanes 1 and 3, respectively), but not mutant (lanes 2 and 4, respectively) extracts.

(B) Western blots of IPs using DPY-26 antibodies and nuclear extracts from wild-type or *dpy-26* mutant animals. DPY-26 antibodies immunoprecipitate DPY-26 protein (lane 1, doublet) and MIX-1 from wild-type embryonic (lane 2) or mixed-staged (lane 3) extracts, but not from mixed-staged extracts lacking DPY-26 (lane 4). MIX-1 antibody IP probed with MIX-1 antibodies (lane 5).

(C) Western blots of IPs using MIX-1 antibodies and nuclear extracts from wild-type or *dpy-27* mutant embryos that were probed with either MIX-1 or DPY-27 antibodies. MIX-1 antibodies immunoprecipitate both MIX-1 (lane 1) and DPY-27 (lane 3) from wild-type embryonic extracts, but only MIX-1 from *dpy-27* mutant extracts (lane 2 compared to lane 4). Purified FLAG-tagged DPY-27 protein (lane 5).

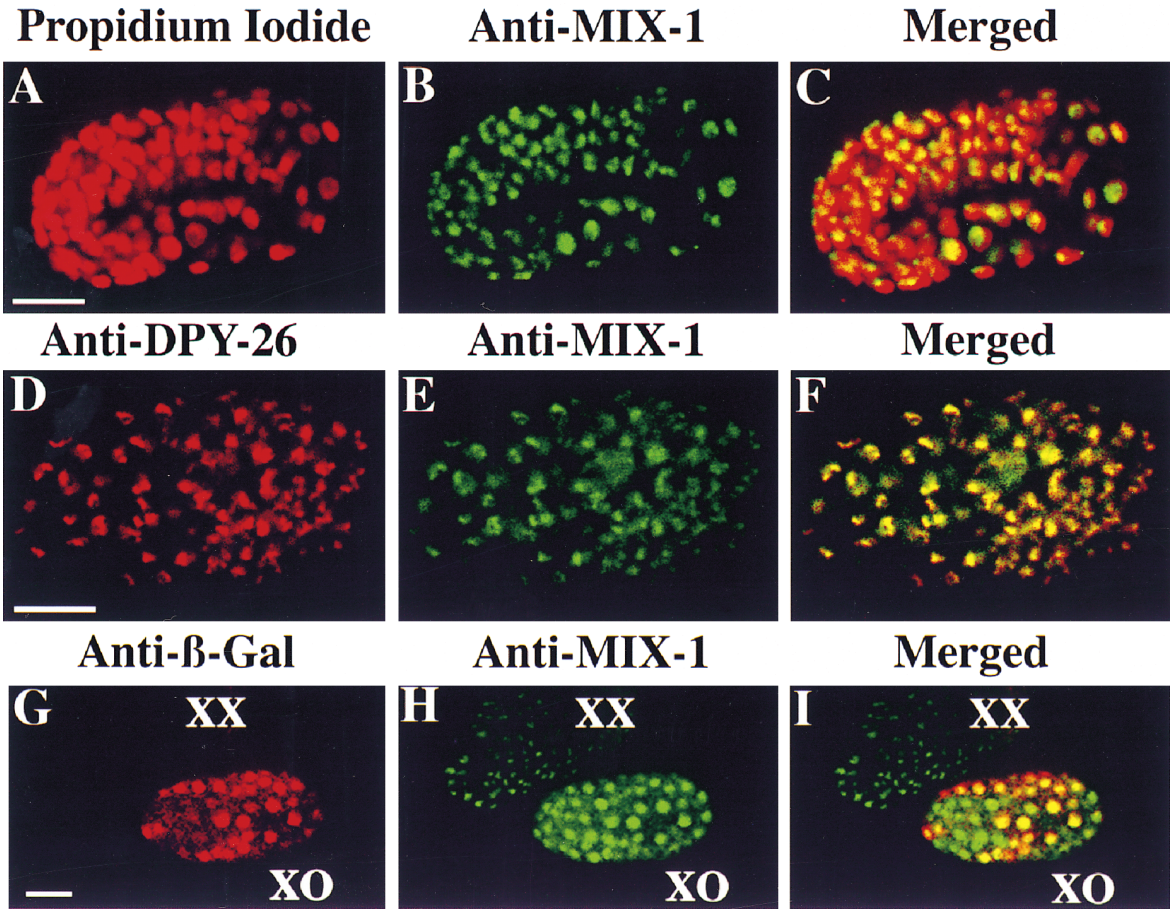


Figure 2. MIX-1 Is Associated with the X Chromosomes of Hermaphrodite but Not Male Embryos

All panels are false-color confocal images.

(A–C) Wild-type XX embryo (~500 cells) costained with propidium iodide (PI), a DNA-intercalating dye (A; red), and MIX-1 antibodies (B; green). The characteristic subnuclear, punctate pattern of MIX-1 appears yellow in the merged image (C).

(D–F) Wild-type XX embryo (~500 cells) costained with DPY-26 (D; red) and MIX-1 (E; green) antibodies. The areas of greatest MIX-1 intensity are coincident with the X-localized DPY-26 protein (F; yellow), showing that MIX-1 associates with X chromosomes.

(G–I) A pair of XX (>500 cells) and XO (~200 cells) *him-8 yIs2(xol-1::lacZ)* embryos costained with β-galactosidase (G; red) and MIX-1 (H; green) antibodies. Merged image in (I). The *xol-1::lacZ* fusion is expressed exclusively in XO embryos and in most but not all cells. Scale bars, 10 μm.

## Results

### A Biochemically Defined Component of the Dosage Compensation Complex Is a Homolog of Proteins Involved in Chromosome Condensation and Segregation

The similarity of DPY-27 to one subclass of SMC proteins (XCAP-C), together with the knowledge that SMC proteins of different subclasses form heterodimers, prompted us to determine if either the ~150 kDa or the ~160 kDa uncharacterized component of the dosage compensation complex belongs to the SMC family (Chuang et al., 1996). We cloned a *C. elegans* homolog of the XCAP-E subclass and raised antibodies against portions of the predicted 1245 amino acid protein called MIX-1 (see Experimental Procedures). The experiments described below showed that MIX-1 migrates in SDS-PAGE at the same position as the ~150 kDa component, and MIX-1 is essential for X chromosome dosage compensation and chromosome segregation during mitosis.

To test whether MIX-1 forms a stable complex with DPY-26 and DPY-27 in vivo, we performed immunoprecipitation experiments using affinity-purified antibodies to DPY-27 or DPY-26 and nuclear extracts from wild-type and mutant *C. elegans* embryos. The resultant immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with affinity-purified antibodies (Figure 1). As expected, DPY-27 antibodies immunoprecipitated DPY-27 from wild-type nuclear extracts but not from *dpy-27* mutant extracts (Figure 1A, lanes 1 and 2). The DPY-27 antibodies also immunoprecipitated MIX-1 from wild-type extracts but not from the control *dpy-27* mutant extracts (Figure 1A, lanes 3 and 4), demonstrating that DPY-27 and MIX-1 form a stable complex. This DPY-27/MIX-1 interaction is robust and withstands high stringency washes up to 1M KCl (data not shown). MIX-1 was also immunoprecipitated with DPY-26 antibodies in a DPY-26-dependent manner (Figure 1B). In a reciprocal

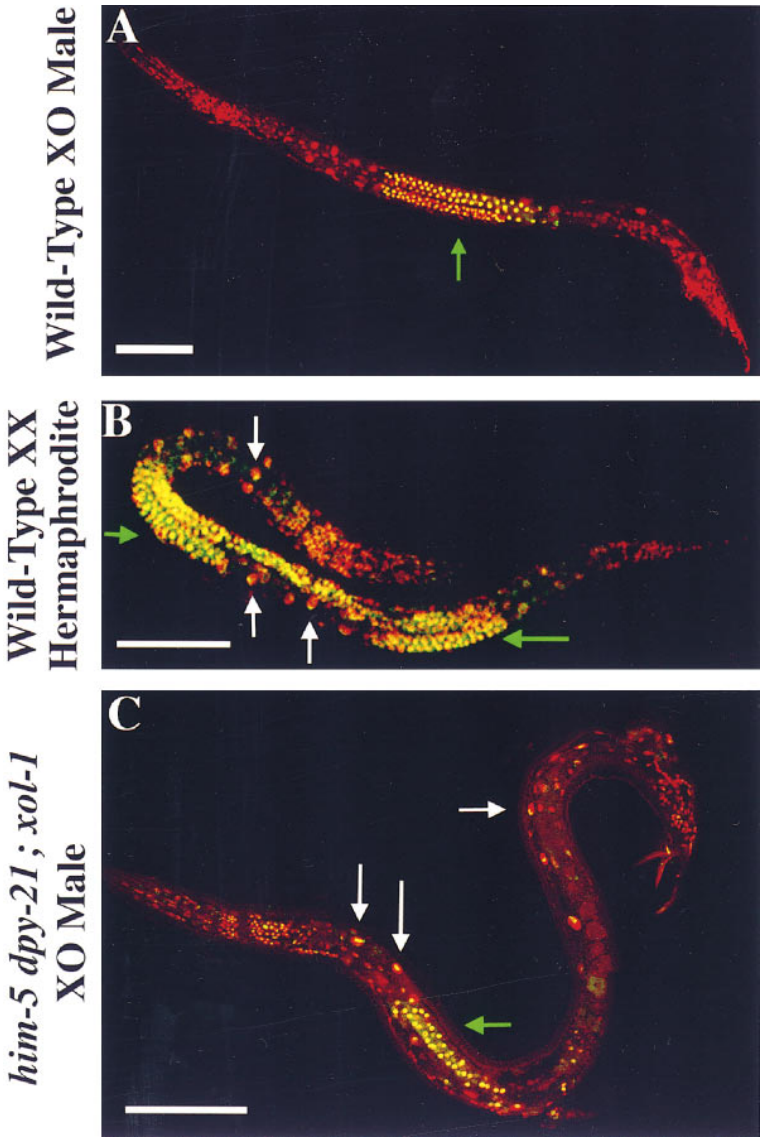


Figure 3. MIX-1 Is Absent From Nondividing Somatic Cells in Wild-Type Males and Is Mislocalized to X in *xol-1* Mutant Males

All panels are false-color confocal images. (A) A merged image of a wild-type adult male costained with propidium iodide (PI, red), and MIX-1 antibodies (green). MIX-1 is absent from all nondividing somatic cells but associates with all mitotic chromosomes and pachytene meiotic chromosomes within the one-armed gonad (green arrow).

(B) A merged image of a wild-type adult hermaphrodite costained with PI (red) and MIX-1 (green) antibodies. The yellow subnuclear dots (white arrows) represent the MIX-1 X chromosome staining in somatic nuclei. MIX-1 also associates with all mitotic chromosomes and pachytene meiotic chromosomes of the two-armed gonad (green arrows). A role for MIX-1 in meiosis has not yet been defined.

(C) A merged image of a *him-5(e1490) dpy-21(e428); xol-1(y9)* XO male costained with PI (red) and MIX-1 antibodies (green). The subnuclear, punctate MIX-1 staining pattern is similar to that of wild-type hermaphrodites, indicating that MIX-1 is inappropriately localized to X. In this one-armed gonad, mitotic and meiotic chromosome staining (green arrow) is normal. Scale bars, 100 μm.

experiment, MIX-1 antibodies immunoprecipitated DPY-26 (data not shown), and MIX-1 and DPY-27 (Figure 1C, lanes 1 and 3) from wild-type nuclear extracts, confirming the existence of the DPY-27/MIX-1/DPY-26 complex. In addition, cofractionation of DPY-26, DPY-27, and MIX-1 was observed through the sequential cation and anion exchange chromatography used for the partial purification of the dosage compensation complex in Chuang et al. (1996) (data not shown). Together, these biochemical data implicate the XCAP-E homolog, MIX-1, as the ~150 kDa component of the dosage compensation complex.

### During Interphase, MIX-1 Is Localized to the X Chromosomes of Wild-Type XX but Not XO Animals

If MIX-1 functions as a member of the dosage compensation complex in vivo, two expectations should be met concerning the distribution of MIX-1 in embryos. MIX-1 should become localized to the X chromosomes of XX embryos at approximately the 30-cell stage, when dosage compensation is activated, and the X localization should be hermaphrodite-specific. In immunofluorescence experiments using MIX-1 antibodies, we observed that hermaphrodite (XX) embryos at or beyond the 30-cell stage consistently exhibit punctate, subnuclear foci of intense MIX-1 staining (Figures 2A–2C). These areas of increased MIX-1 intensity are coincident with the staining observed with DPY-26 antibodies (Figures 2D–2F), indicating that MIX-1 localizes to the X chromosomes. In young dosage-compensating XX embryos, diffuse nuclear staining is also seen, reflecting the mitotic function of MIX-1 (see below).

The sex specificity of the X chromosome localization was addressed using a reporter transgene in which the male-specific *xol-1* promoter directs expression of the *lacZ* gene. XO but not XX embryos carrying this reporter express β-galactosidase (Rhind et al., 1995). Costaining

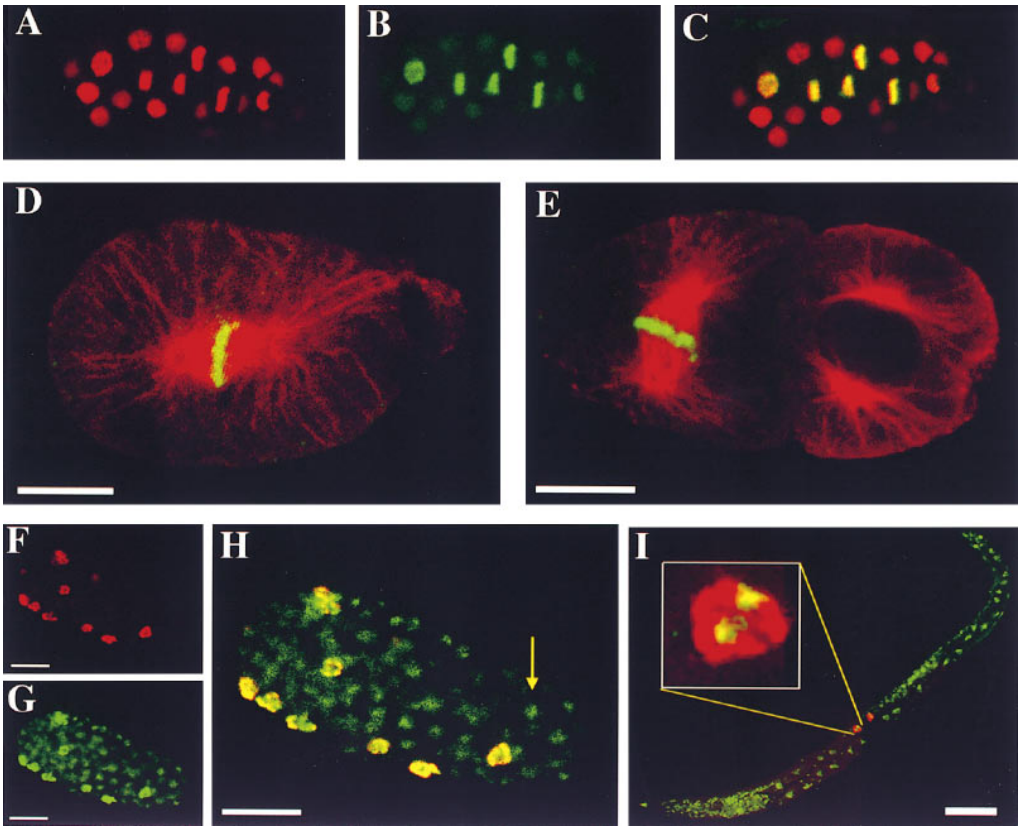


Figure 4. MIX-1 Localizes to All Chromosomes in Every Mitotic Cell

All panels are false-color confocal images.

(A–C) Wild-type XX embryo costained with PI (A) and anti-phosphorylated histone H3 antibody (B). Merged image in (C). Histone H3 is phosphorylated from prometaphase to telophase, making it an ideal marker for the M phase of the cell cycle (Hendzel et al., 1997). In this embryo, phosphorylated histone H3 is present in five nuclei, four of which are in metaphase (bar-shaped chromosomes) and one in prometaphase.

(D) Merged image of a wild-type one-celled XX embryo costained with anti-α-tubulin (red) and MIX-1 (green) antibodies, showing that MIX-1 is coincident with the mitotic chromosomes on the metaphase plate.

(E) Merged image of a wild-type two-celled XX embryo stained with anti-α-tubulin (red) and MIX-1 (green) antibodies. The AB cell (left) is in metaphase, but the P1 cell (right) has not yet initiated mitosis. No MIX-1 staining is evident in the P1 cell, which divides 3 min after AB.

(F–H) An (~500-cell) embryo costained with anti-phosphorylated histone H3 (F; red) and anti-MIX-1 (G; green) antibodies. Merged image in (H). MIX-1 is localized to all of the chromosomes in mitotic cells but is localized only to X in interphase nuclei (yellow arrow).

(I) Merged image of an L1 larvae costained with anti-phosphorylated histone H3 (red) and anti-DPY-26 (green) antibodies. DPY-26 (yellow dots) is only associated with X among the prometaphase mitotic chromosomes.

Scale bars, 10 μm in (A–H) and 25 μm in (I).

experiments with antibodies to β-galactosidase and MIX-1 revealed that the embryos with no β-galactosidase staining showed the subnuclear, punctate MIX-1 staining pattern characteristic of XX embryos, and embryos with strong β-galactosidase staining had a uniform nuclear MIX-1 staining pattern that is diminished by the 1 1/2–fold stage of embryogenesis (>500 cells), when most cell divisions are complete (compare XX to XO, Figures 2G–2I). Therefore, MIX-1 is produced in both sexes but localizes to the X chromosomes of XX but not XO embryos during interphase. To confirm that the X localization of MIX-1 is hermaphrodite-specific, we stained adult XX and XO animals with MIX-1 antibodies. Subnuclear, punctate MIX-1 staining was apparent in the somatic cells of XX hermaphrodites (Figure 3B), but no MIX-1 staining was visible in the somatic cells of XO males (Figure 3A). We confirmed that the MIX-1 staining in hermaphrodites was X chromosome–specific by costaining adults with DPY-26 and MIX-1 antibodies (data not shown).

To address how MIX-1's function in dosage compensation is sex-specifically regulated, we determined the effect of a *xol-1* mutation on the localization of MIX-1. *xol-1* is a switch gene that directs the male modes of both sex determination and dosage compensation by negatively regulating the XX-specific *sdc* genes that coordinately control both processes (Miller et al., 1988; Rhind et al., 1995). Mutations in *xol-1* kill XO animals by causing the dosage compensation complex to associate with the single X chromosome and thereby reduce X gene expression (Chuang et al., 1994; Lieb et al., 1996; Davis and Meyer, 1997). We stained *xol-1* XO adult males

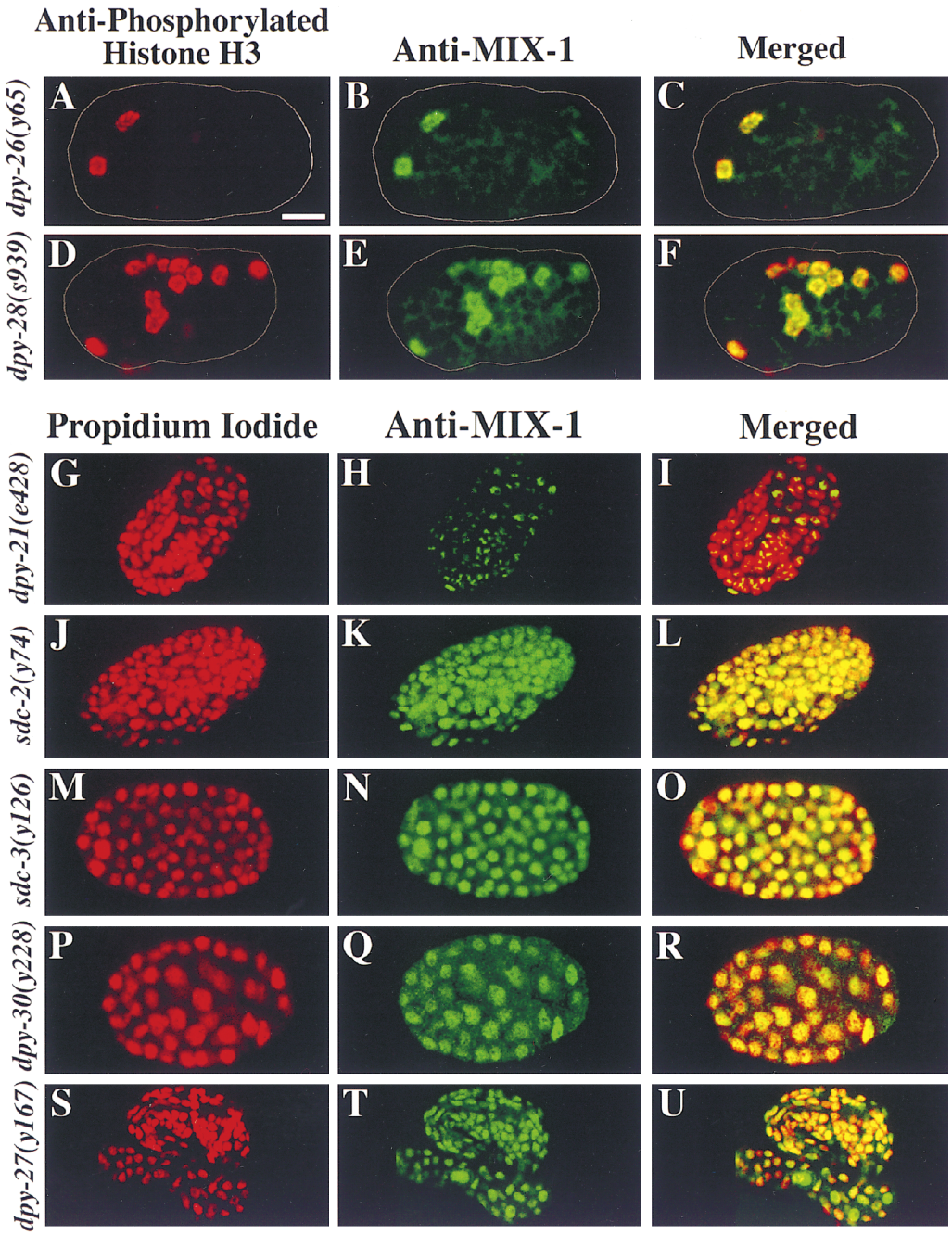


Figure 5. The X Localization of MIX-1 Is Dependent on Dosage Compensation Genes

All panels are false-color confocal images.

(A–C) A *dpy-26* mutant embryo costained with anti-phosphorylated histone H3 (A) and anti-MIX-1 (B) antibodies. (C) Merged image.

(D–F) A *dpy-28* mutant embryo costained with anti-phosphorylated histone H3 (D) and anti-MIX-1 (E) antibodies. (F) Merged image. In both mutants, MIX-1 is absent from the nuclei of cells in interphase but is abundant in cells undergoing mitosis. Faint cytoplasmic staining is observed occasionally in nonmitotic cells (E). The edges of each embryo (A–F) are marked with a white line.

(G–U) Dosage compensation–defective embryos costained with PI (left column) and MIX-1 antibodies (center column). Merged images (right column). The X chromosome localization of MIX-1 is unaffected by mutation of *dpy-21* (G–I). Mutations in the dosage compensation genes *sdc-2* (J–L), *sdc-3* (M–O), *dpy-30* (P–R), or *dpy-27* (S–U) prevent the association of MIX-1 with X. Scale bar, 10 μm.

whose death had been suppressed by a mutation in *dpy-21*, a dosage compensation gene required for the function but not the X association of the dosage compensation complex (Chuang et al., 1996). In these adults, MIX-1 colocalized with X (compare Figures 3B and 3C), indicating that in wild-type males, *xol-1* prevents MIX-1 from associating with X. In conclusion, three results indicate that MIX-1 plays a role in dosage compensation:

the biochemical demonstration of a protein complex containing MIX-1 and the dosage compensation proteins DPY-26 and DPY-27, the hermaphrodite-specific localization of MIX-1 to the X chromosomes of wild-type somatic cells, and the ectopic localization of MIX-1 to the male X chromosome in *xol-1* mutants.

### MIX-1 Associates with All Chromosomes at Each Mitosis in Both Males and Hermaphrodites

In many organisms, the SMC proteins act during mitosis to ensure the proper condensation and segregation of chromosomes. To determine whether MIX-1 functions outside the context of dosage compensation in *C. elegans*, we analyzed the distribution of MIX-1 throughout the cell cycle using MIX-1 antibodies and a mitotic-cell-specific antibody (Figures 4A–4C) directed against the phosphorylated form of histone H3 (Hendzel et al., 1997). In young embryos, prior to the initiation of dosage compensation ($<$30 cells), MIX-1 is localized to all chromosomes during mitosis (Figures 4D and 4E). In older wild-type XX embryos that have activated dosage compensation, MIX-1 is localized to X in interphase cells; it then appears to accumulate to a higher level in mitotic cells, where it associates with all chromosomes (Figures 4F–4H). In contrast, the dosage compensation proteins DPY-26 and DPY-27 are associated exclusively with the X chromosome during mitosis (Figure 4I and data not shown). These results suggest that MIX-1 has a functional role in mitosis and that it is regulated in a cell cycle–dependent manner.

The apparent cell-cycle regulation becomes obvious as early as the two-cell stage, when the anterior AB cell divides just ahead of the posterior P1. In embryos of this age, MIX-1 protein can be seen in AB, but not in P1 (Figure 4E). After mitosis, MIX-1 staining becomes more diffuse and less intense, only to increase in intensity just prior to the next mitosis. This apparent oscillation in MIX-1 protein levels is obscured by the accumulation of MIX-1 protein in the interphase nuclei of embryos ($>$30-cell stage) undergoing rapid cell divisions. However, at a time after most embryonic cell divisions have occurred, cell cycle regulation of MIX-1 becomes noticeable once again (Figures 4F–4H). Mitotic cells exhibit intense MIX-1 staining, and interphase cells exhibit weaker X chromosome–specific staining. The cell-cycle difference in the accumulation of MIX-1 is also obvious in specific dosage compensation mutants (see below) and in wild-type males, where the dosage compensation process is not active. For example, MIX-1 protein is virtually absent from male somatic cells by the fourth larval stage (L4), when most cell division is complete. However, intense MIX-1 staining is seen in the L4 male tail cells that divide and differentiate as part of sexual maturation (data not shown). Together, these results suggest that MIX-1 protein levels increase prior to each mitosis and that the autosome-associated MIX-1 protein is degraded upon the completion of mitosis. The association of MIX-1 with mitotic chromosomes and the cell-cycle dependence of MIX-1 levels suggest that MIX-1 plays a role in mitosis that is independent of its role in dosage compensation.

### The X Chromosome Localization of MIX-1 but Not the Mitotic-Chromosome Localization Is Conferred by Genes that Control Dosage Compensation

How are the roles of MIX-1 in the two separate processes of mitosis and X chromosome dosage compensation specified? We addressed this issue by examining the impact of mutations in dosage compensation genes on the localization of MIX-1 to mitotic chromosomes and to X chromosomes during interphase (see Experimental Procedures). Mutations in all the dosage-compensation *dpy* or *sdc* genes except *sdc-1* and *dpy-21* cause an elevation in X chromosome expression that kills $>$95% of XX animals (reviewed in Cline and Meyer, 1996). These mutations have no effect on mitosis.

We found that in *dpy-26* and *dpy-28* mutants, MIX-1 is completely absent from interphase nuclei; only faint cytoplasmic staining is visible (Figures 5A–5F). Despite the apparent overall reduction of MIX-1 levels, a variable subset of cells in some *dpy-26* or *dpy-28* embryos displayed intense nuclear MIX-1 staining. By costaining the mutant embryos with MIX-1 antibodies and phosphorylated histone H3 antibodies, we showed that the only cells expressing MIX-1 were mitotic cells or cells just entering mitosis (Figures 5A–5F). These results highlight the cell-cycle dependence of MIX-1 accumulation and suggest that the dosage compensation genes *dpy-26* and *dpy-28* are essential for the stability of MIX-1 when it functions in dosage compensation, but they have no major effect on the mitotic function of MIX-1.

In embryos that lack any one of the genes (*sdc-2*, *sdc-3*, and *dpy-30*) that are required for the X chromosome localization of the dosage compensation complex, MIX-1 was not localized to X but was distributed diffusely throughout the interphase nuclei (Figures 5J–5R). MIX-1 also requires DPY-27 for its localization to X, as does DPY-26 (Figures 5S–5U). The MIX-1 staining pattern observed in these mutant embryos was indistinguishable from that of wild-type XO embryos. In contrast, these genes are not required for the increase in MIX-1 protein levels during mitosis or for the localization of MIX-1 to mitotic chromosomes (data not shown). Consistent with previous findings that mutations in *dpy-21* and *sdc-1* have no effect on the localization of the dosage compensation complex to X, we observed that a null mutation in either of these genes also has no effect on the localization of MIX-1 to X (Figures 5G–5I, data not shown). In sum, these observations demonstrate that the X localization and interphase stability of MIX-1 are dependent on genes required for dosage compensation, and that MIX-1 thus behaves as other members of the dosage compensation complex in vivo. However, the dosage compensation mutations have no obvious effect on the mitotic function of MIX-1, indicating that the roles of MIX-1 in mitosis and dosage compensation are specified independently.

### MIX-1 Is Encoded by the Essential Gene *let-29*

To determine whether MIX-1 plays an essential role in both X chromosome dosage compensation and mitotic chromosome segregation in vivo, we obtained and characterized mutants defective in MIX-1. Our search for

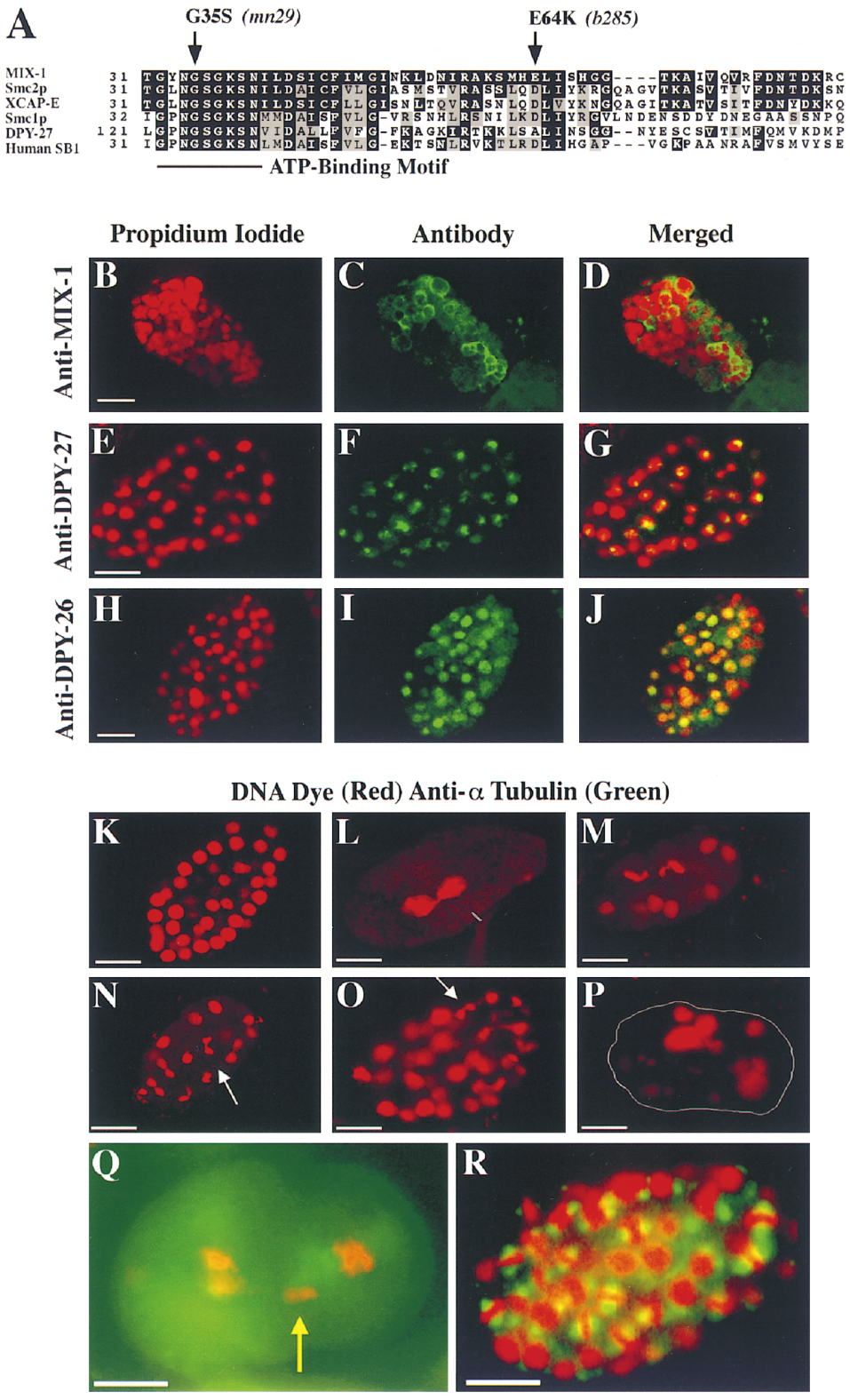


Figure 6. MIX-1 Mutations Cause Dosage Compensation Defects and Mitotic Defects

(A) Similarities among the amino-terminal regions of MIX-1 and selected SMC homologs. The first three proteins, including MIX-1, belong to the XCAP-E subclass and the last three to the XCAP-C subclass of SMC family members. Arrows mark the locations of *mix-1* mutations.

(B–J) False-color confocal images of homozygous *mix-1(b285)* embryos from *mix-1(b285)* homozygous mothers. Embryos were costained with PI (left column, red) and antibodies (green, center column) to MIX-1 (C), DPY-27 (F), or DPY-26 (I). Merged images (right column). (B–D) MIX-1 is cytoplasmically localized in virtually all *mix-1(b285)* embryos. (E–G) The X localization of DPY-27 appears nearly wild type. (H–J) The

*mix-1* mutations focused on mutations lethal to both sexes for two reasons: mitosis is an essential process, and *mix-1* alleles were not recovered in genetic screens for dosage compensation mutations that cause sex-specific lethality. We first determined the position of *mix-1* relative to genetically defined chromosomal deficiencies using a PCR strategy (Experimental Procedures). We then asked whether a wild-type *mix-1* gene could rescue the lethal phenotype caused by mutations in the genes located in that interval.

*mix-1* mapped to an interval that coincides with the genetic map position of four candidate genes defined by mutations that cause either recessive lethal (*let*) or maternal-effect lethal (*mel*) phenotypes (*let-21*, *let-29*, *mel-13*, and *ooc-1)*. To assay whether the wild-type *mix-1* gene could rescue the lethality caused by mutations in any of these genes, a *mix-1* genomic DNA–cDNA fusion construct (pMRA2) was made and injected into the gonads of balanced heterozygous mutant strains (Experimental Procedures). Although *unc-4 let-29(mn29)* homozygous mutants normally die at the L1 or L2 larval stage, we recovered Unc adults from the transgenic progeny of our injected animals, indicating that *mix-1* might rescue the larval lethality of *let-29*. No such rescue was observed for the other candidate genes. However, all the transformed F1 animals were sterile, and attempts to maintain stably transformed lines of *mix-1* in either mutant or wild-type worms failed.

To confirm that *let-29* encodes the MIX-1 protein, we sequenced the *mix-1* gene from *let-29* homozygous mutants. The presumed null allele *let-29(mn29)* (Herman, 1978) has a G-to-A transition at nucleotide position 103, resulting in a glycine to serine change at amino acid position 35 (Figure 6A). The glycine at this position is within the ATP-binding motif shown to be essential for SMC protein function. The glycine is conserved in all SMC proteins ($>$25) and most ATP-binding motifs. A partial-loss-of-function allele, *let-29(b285)* (Kemphues et al., 1988), which causes maternal-effect embryonic lethality, has a G-to-A transition at nucleotide 190, causing a glutamic acid to lysine change at amino acid 64 (Figure 6A). A charge reversal at this residue is likely to have a deleterious effect on protein function, since a negatively charged amino acid, either glutamic acid or aspartic acid, is found at this position in most SMC proteins (Figure 6A).

Further indication that the charge reversal in *let-29(b285)* affects the function of MIX-1 was revealed by the abnormal cytoplasmic distribution of MIX-1 in homozygous *let-29(b285)* embryos stained with MIX-1 antibodies (Figures 6B–6D). Only rarely was MIX-1 correctly associated with mitotic chromosomes in these mutants. Based on the rescue obtained in the injection experiments, the DNA sequence changes found in *let-29* homozygous animals, and the distribution of MIX-1 protein in mutant embryos, we conclude that MIX-1 is encoded by *let-29*. We therefore changed the name of the *let-29* gene to *mix-1*.

### Mutations in *mix-1* Cause Defects in Mitotic Chromosome Segregation, and Possibly Condensation, in Both Sexes

We observed that *mix-1* mutants exhibit defects in the segregation of mitotic chromosomes. Animals homozygous for null alleles of *mix-1* survive for extended periods as arrested L1 or L2 larvae, presumably because the maternal contribution of wild-type MIX-1 suffices for embryonic development. The arrested *mix-1(mn29)* larvae, having depleted the maternal MIX-1 activity, exhibit variable pleiotropic defects concordant with abnormal nuclear division (see Experimental Procedures). Mitotic defects are most striking in embryos from mothers that carry the homozygous *mix-1(b285)* mutation. First-generation hermaphrodites homozygous for the partial-loss-of-function *mix-1(b285)* mutation survive to adulthood, but 100% of their offspring die as embryos because they lack the wild-type maternal contribution of MIX-1 (Kemphues et al., 1988). These dying *mix-1(b285)* embryos display extensive anomalies in chromosome behavior (Figures 6L–6P), with defects in chromosome segregation apparent as early as the two-cell stage (Figure 6L). In these mutants, chromatin bridges are a common feature of chromosomes attempting to separate at anaphase, suggesting a persistence of sister chromatid adhesion or the unresolved entanglement of postreplication DNA (Figures 6L–6O). In every embryo, interphase nuclei appear swollen and irregularly shaped compared to those in wild-type embryos (compare Figures 6M–6P with Figure 6K). In some cases, massive aggregates of DNA occupied large portions of the embryo (Figure 6P), consistent with either gross chromosome condensation defects or multiple rounds of DNA replication without cell division (endoreduplication). In addition, *mix-1(b285)* embryos fail to segregate chromosomes properly, as demonstrated by lagging chromosomes (Figure 6M) and chromosomes that have not migrated toward the spindle poles (Figure 6Q). The chromosome segregation defect is not likely to be caused by a spindle defect because the morphology of mitotic spindles in *mix-1(b285)* embryos was indistinguishable from that in wild-type embryos (data not shown). We

X localization of DPY-26 is impaired; only an occasional cell exhibits the subnuclear punctate staining characteristic of X chromosome localization.

(K–P) Embryos stained with PI (red). (K) Wild-type embryo. (L–P) *mix-1(b285)* homozygous embryos from *mix-1(b285)* homozygous mothers. Serious defects in chromosome structure are apparent. (L) a chromatin bridge prevents the two sets of chromosomes from separating. (M) A broken chromatin bridge. (N–O) Chromatin bridges (arrows) during later embryogenesis. (P) DNA in large, irregularly shaped masses. The edges of this embryo are marked with a white line. (Q)–(R) are false-color fluorescence photomicrographs of *mix-1(b285)* embryos stained with the DNA-intercalating dye, DAPI (red) and $\alpha$-tubulin antibodies (green).

(Q) A two-celled mutant embryo in which a chromosome (yellow arrow) that had not segregated in the first mitotic division is on the wrong side of both mitotic spindles.

(R) A typical ($\sim$300 cell) mutant embryo in which almost every cell arrested in mitosis, as indicated by the unusual number of brightly staining spindle poles and the metaphase appearance of DNA. Only a small fraction of cells in wild-type embryos show such staining. Scale bars, 10 $\mu$m.

Table 1. *mix-1* Mutants Are Defective in Dosage Compensation

| Parental Genotypes | Number of Progeny | | | | |
|---|---|---|---|---|---|
| | Males [homozygous *sdc-3*(Tra) *unc-76*] | Hermaphrodites [homozygous *sdc-3*(Tra) *unc-76*] | Arrested *mix-1* Homozygotes | *mnC1* homozygotes | Wild Type |
| *unc-4 mix-1(mn29)*/++; *sdc-3*(Tra) *unc-76*/++ XX | 13 (3.3%) | 382 (96.7%) | 705 | | 1468 |
| *mnC1*/+; *sdc-3*(Tra) *unc-76*/++ XX | 318 (86.9%) | 48 (13.1%) | | 539 | 1279 |

The involvement of *mix-1* in dosage compensation was assessed using a genetic assay in which dosage compensation mutations are expected to cause a transformation of sexual fate. Normally, *sdc-3*(Tra)/*sdc-3*(Tra) XX animals from *sdc-3*(Tra)/+ XX mothers are males. However, if *m* is a mutation in a dosage compensation gene, *m*/+; *sdc-3*(Tra)/*sdc-3*(Tra) XX animals from *m*/+; *sdc-3*(Tra)/+ XX mothers are hermaphrodites. In our experiment, the *unc-4 mix-1(mn29)*/++; *sdc-3*(Tra) *unc-76*/++ parents and the *mnC1*/+; *sdc-3*(Tra) *unc-76*/++ control parents were generated as described in the Experimental Procedures. These individual L4 animals were picked to plates and transferred every 12 hr. Their genotype was inferred from their progeny. Dpy Unc animals signified a *mnC1*/+ parent. Unc-4-arrested L1-L2 animals signified a *mix-1*/+ parent. In the assay, homozygous *sdc-3*(Tra) *unc-76* animals were identified by their Unc-76 phenotype, which is distinguishable from the Unc-4 phenotype. Unc-76 animals were scored as males if they had male tail structures. Percentages refer to the number of males or hermaphrodites in the Unc-76 population. The *mix-1* mutation suppresses *sdc-3*(Tra) to the same degree as a *dpy-27* mutation (see DeLong et al., 1993).

conclude that wild-type MIX-1 function is required for the segregation and possibly the condensation of chromosomes at mitosis.

Many *mix-1(b285)* embryos that live to later stages of embryogenesis have an unusually large number of cells arrested in metaphase (Figure 6R). In contrast, younger embryos attempt cell division despite having highly abnormal chromosome morphology. Therefore, a checkpoint specific to older *C. elegans* embryos may act to stop progression through the cell cycle in response to chromosomal abnormalities induced by lack of functional MIX-1.

### *mix-1* Is Essential for Dosage Compensation

Analysis of *mix-1* mutants revealed that the dosage compensation complex does not form properly on X chromosomes. The effect on complex formation was assessed by staining the progeny of hermaphrodites homozygous for the partial-loss-of-function mutation, *mix-1(b285)*, with antibodies to DPY-26 and to DPY-27 (Figures 6E–6J). While the DPY-27 protein was localized to X in these mutants despite gross abnormalities in nuclear structure (Figures 6E–6G), the distribution of DPY-26 protein was abnormal. DPY-26 protein often occupied the entire volume of the nucleus and was only rarely associated specifically with the X chromosomes (Figures 6H–6J), suggesting that the dosage compensation mechanism is defective.

We further demonstrated the involvement of *mix-1* in dosage compensation using a genetic assay that detects disruptions in dosage compensation caused by the loss of only one of the two wild-type copies of a dosage compensation gene. This assay for dominant effects of *mix-1* was essential because animals homozygous for null alleles of *mix-1* are dead. The assay is based on the observation that disruptions in dosage compensation can cause reversal of sexual fate (DeLong et al., 1993). Specifically, the *sdc-3(y52*Tra) sex determination mutation—referred to as *sdc-3*(Tra)—causes XX animals (normally hermaphrodites) to develop as males, and this masculinization can be reversed in a dominant manner by loss-of-function dosage compensation mutations. For example, when *m* is a mutation in a dosage compensation gene, *m*/+; *sdc-3*(Tra)/*sdc-3*(Tra) animals are hermaphrodites, whereas +/+; *sdc-3*(Tra)/*sdc-3*(Tra) animals are males. This phenomenon has been observed with mutations in all genes known to implement dosage compensation but not regulate sex determination (*dpy-21*, *dpy-26*, *dpy-27*, and *dpy-28*). The effect has been correlated with an elevation in X chromosome gene expression and has never been observed with mutations that have no effect on gene expression (DeLong et al., 1993). We created *mix-1(mn29)*/+; *sdc-3*(Tra)/*sdc-3*(Tra) animals and scored their sexual phenotype (Table 1). While 87% of the +/+; *sdc-3*(Tra)/*sdc-3*(Tra) control animals were male, only 3% of the *mix-1(mn29)*/+; *sdc-3*(Tra)/*sdc-3*(Tra) animals were male and 97% were hermaphrodite, demonstrating that *mix-1(mn29)* has a dominant suppressing effect on the masculinization caused by *sdc-3*(Tra). We conclude that mutation of *mix-1* causes an elevation of X chromosome gene expression as the result of a dosage compensation defect.

## Discussion

We have demonstrated that an essential component of the mitotic machinery in *C. elegans* also regulates the expression of X chromosome genes during interphase. This protein, MIX-1, is an integral component of the dosage compensation complex that associates specifically with hermaphrodite X chromosomes to reduce their gene expression. MIX-1 also accumulates to high levels at each mitosis and associates with all mitotic chromosomes to ensure their proper segregation. The role of MIX-1 in this vital cellular process precluded its identification in previous searches for dosage compensation genes that exploited the sex-specific action of dosage compensation (reviewed in Cline and Meyer, 1996).

MIX-1 belongs to the SMC family of proteins shown to be essential for mitotic chromosome condensation and segregation in yeast and frogs (reviewed in Koshland and Strunnikov, 1996). In neither organism have these proteins been implicated in gene regulation. The involvement of MIX-1 in *C. elegans* mitosis confirms the evolutionary conservation of SMC protein function and

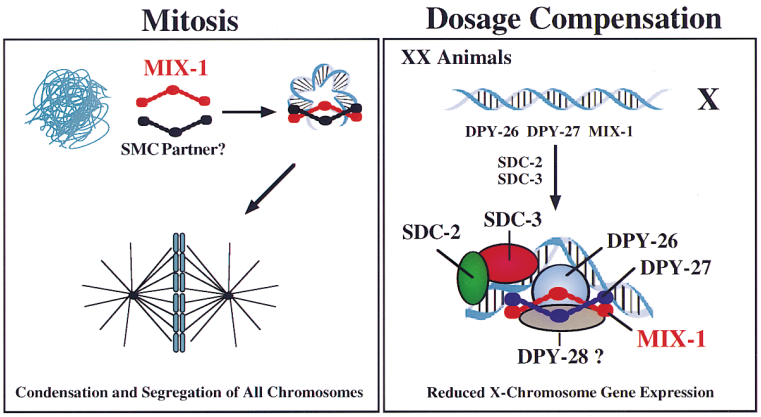


Figure 7. A Model for MIX-1 Function

MIX-1 has two separate, but perhaps related, functions. During mitosis, MIX-1 associates with all chromosomes in both sexes and acts with unidentified mitotic factors to ensure proper chromosome condensation and segregation. During interphase, MIX-1, in conjunction with dosage compensation–specific proteins, localizes to the X chromosomes of XX hermaphrodites and reduces X chromosome gene expression. Analysis of *C. elegans* dosage compensation may reveal how changes in the higher order structure of interphase chromosomes perturb gene expression and thereby clarify the role of interphase chromosome structure in the general expression of genes.

suggests that the participation of SMC proteins in this aspect of gene regulation is a more recent adaptation. In addition, the finding that mitosis and dosage compensation share a common component indicates that the mechanisms underlying these processes are closely related and likely to involve the organization of higher order chromosome structure.

### Recruitment of a Mitotic Protein to the Dosage Compensation Process

We can infer from our results that the *C. elegans* dosage compensation process evolved by recruiting components used in other chromosome behaviors to the new task of fine-tuning X chromosome expression. That is, proteins exclusive to dosage compensation recruited a general mitotic factor to the X chromosomes of hermaphrodites, thereby permitting a chromosome condensation protein to function as a global regulator of gene expression (Figure 7). This inference is reinforced by our finding that the dual roles of MIX-1 in mitosis and dosage compensation are specified independently. MIX-1 requires the activities of other dosage compensation proteins to regulate X chromosome gene expression, but these genes are not required for the mitotic function. DPY-26 and DPY-28 are needed for the stability of MIX-1 in interphase cells but not in mitotic cells. The MIX-1 protein that forms a complex with the dosage compensation proteins appears to be protected from degradation after mitosis. In addition, the participation of MIX-1 in X chromosome gene regulation is dependent on the dosage compensation proteins SDC-2 and SDC-3, which coordinate the sex-specific association of the dosage compensation complex on X (Chuang et al., 1996; Lieb et al., 1996; Davis and Meyer, 1997). Although MIX-1 cannot associate with interphase X chromosomes in the absence of these proteins, it retains its ability to interact with all mitotic chromosomes and to function normally in mitosis.

MIX-1 may have been recruited to the dosage compensation process through the evolution of the specialized SMC protein, DPY-27, which plays no role in chromosome segregation and associates only with X chromosomes. MIX-1 cannot associate with interphase X chromosomes in the absence of DPY-27, but DPY-27 retains its ability to associate with X despite the reduction of MIX-1 activity caused by the *mix-1(b285)* mutation. DPY-27 is in turn directed to X by the hermaphrodite-specific proteins SDC-2 and SDC-3. DPY-27 and MIX-1 belong to two different subclasses of SMC proteins known to form heterodimers during mitosis: XCAP-C and XCAP-E, respectively. These combined results suggest that MIX-1's role in dosage compensation is conferred by its association with a dosage compensation–specific protein and that the different functions of a particular SMC protein can be specified by its protein partners within a single cell. It follows that an SMC protein might be used in multiple protein complexes for separate cellular functions in other organisms. Our results also suggest that DPY-27 may have evolved to recruit MIX-1 to X, and they predict that MIX-1 may interact with a more conventional SMC partner for its role in mitosis (Figure 7).

Our finding indicates that the evolution of *C. elegans* dosage compensation did not require the de novo creation of all its components. A requirement for dosage compensation arises with the evolution of chromosome-based mechanisms of sex determination that cause a difference in sex chromosome dose between males and females. Since sex-determining mechanisms evolve rapidly, dosage compensation mechanisms must evolve equally rapidly to prevent sex-specific lethality (Charlesworth, 1996). The recruitment of MIX-1 to dosage compensation from the ancient process of mitosis suggests how dosage compensation was able to coevolve with sex determination and suggests that the evolutionary leap that created the dosage compensation machinery may not be as large as would otherwise appear.

### Parallels between a Dosage Compensation Complex and a Mitotic Chromosome Complex

The involvement of MIX-1 in both dosage compensation and mitosis justifies a comparison between the dosage compensation complex in *C. elegans* and the complex of structural proteins in *X. laevis* that drives mitotic chromosome condensation in vitro, the 13S condensin complex (Hirano et al., 1997). The condensin complex includes the SMC proteins, XCAP-C and XCAP-E, and three other proteins, one of which (XCAP-H) is a homolog of the *Drosophila* Barren protein. Barren, like XCAP-H, is a mitosis-specific protein; it is required for the separation of sister chromatids at anaphase and acts by modulating the activity of topoisomerase II, an enzyme required for chromosome condensation (Bhat et al., 1996). Previous to this work, the only connections between the

complexes were the similarities between DPY-27 and XCAP-C as well as DPY-26 and XCAP-H. The lack of a mitotic function for either DPY protein and the very limited similarity between DPY-26 and Barren homologs made the connection tenuous. The participation of MIX-1, an XCAP-E homolog, in both mitosis and dosage compensation, greatly strengthens the parallels between the dosage compensation complex and the condensin complex and implies that these two complexes will have similar biochemical activities.

It will be important to learn if other members of the dosage compensation complex are similar to members of the condensin complex. It will also be important to learn if the primary roles of SDC-2 and SDC-3 are to recruit the dosage compensation complex specifically to X, or whether these proteins also play an active part in the biochemical activity of the dosage compensation complex when it is associated with X chromosomes. Perhaps the SDC proteins regulate the distribution of the dosage compensation protein complex along X and thereby limit the impact of this complex on the higher order structure of X.

A recent study has demonstrated that the 13S condensin complex introduces positive supercoils into closed circular DNA in an ATP- and topoisomerase I–dependent manner (Kimura and Hirano, 1997). The authors suggested that this change in structure may underlie the compaction of chromatin fibers during mitosis. This activity could be germane to how MIX-1, together with other dosage compensation proteins, might alter the structure of the interphase X chromosomes and thereby reduce the transcription of large blocks of genes. In the case of dosage compensation, DPY-27 may act as a "defective" partner for MIX-1 that limits the extent of condensation (Figure 7). The resulting partial condensation could cause a 2-fold reduction of X chromosome gene expression rather than the complete silencing that occurs during mitosis.

Additional genes necessary for the execution of dosage compensation might also be required for essential cellular processes. By combining a biochemical approach with a genetic screen designed to detect heterozygous mutants with dosage compensation defects, we hope to find such vital genes that will enhance our understanding of the dosage compensation mechanism.

## Experimental Procedures

### Isolation of *mix-1*

A 200 bp fragment of *mix-1* was obtained with PCR using degenerate DNA primers corresponding to conserved carboxy-terminal regions of XCAP-E homologs, ELSGGQR (PT-76, 5′-GARYTIWSIGGIGGICA RMG-3′) and QFIVVSLK (PT-78, 5′-YTTIARIGAIACDATRAAYTG-3′). A partial DNA sequence of *mix-1* from the *C. elegans* genome project revealed that *mix-1* spans the nonoverlapping cosmids R06F6 and M106 of *LG II*. PCR showed that the gap spans 4.5 kb of *mix-1* genomic DNA. We sequenced the largest (yk12b6, 3.6 kb) of four *mix-1* expressed sequence tags (from Y. Kohara) on both strands.

*mix-1* was mapped relative to deficiencies using PCRs on single homozygous mutant embryos. Primers specific to the first exon/intron boundary of *mix-1*, MA15 (5′-CGCAATCACCGGTGAGTA TAG-3′) and the fourth exon, PT90 (5′- CGGAATTCTCATCTCGTTAG GGATTCGAGGTC-3′), were used to create a 1364 bp diagnostic product. Primers specific to the unlinked gene *dpy-26* served as a control for the PCRs. The results were as follows: 15/15 *mnDf90* embryos and 14/14 *mnDf83* embryos deleted *mix-1*; 16/16 *mnDf86* embryos and 10/10 *mnDf57* embryos failed to delete *mix-1*. Thus, *mix-1* resides in the small interval between *mnDf57* and *mnDf86*.

For germline transformation rescue experiments, a clone of the entire *mix-1* gene (pMRA2) was made by fusing genomic DNA with cDNA that bridged the cosmid gap. Specifically, a 4598 bp EagI-XhoI fragment corresponding to nucleotides 35656–40254 of cosmid R06F6 was fused to the *mix-1* cDNA yk12b6 via its XhoI site at nucleotide 40254 (exon 3). The pMRA2 clone (52 ng/ml) was coinjected with the dominant marker *rol-6* (100 ng/μl) (Mello et al., 1991) into the gonads of the four appropriately balanced mutant strains. Partial rescue was only obtained for the *let-29* strains *unc-4(e120) let-29(mn29* or *mn182)/dpy-10 unc-52 mnC1*. For example, one set of injected *let-29* animals produced 96 wild-type Rol F1 adults (balanced heterozygotes) and 52 Unc Rol F1 sterile adults (the rescued *unc-4 let-29* homozygotes), implying rescue of all the transgenic *let-29* F1 mutants. Another set of *let-29* animals injected at a lower concentration of *mix-1* DNA (5 ng/μl) produced 196 wild-type Rol F1 adults and 53 Unc Rol F1 sterile adults.

To determine the DNA sequence changes associated with the *let-29* alleles *mn29* and *b285*, single homozygous mutant worms were picked and subjected to PCR using *mix-1* primers. For each mutation, PCR products from three independent reactions were sequenced on both strands.

### *mix-1* Null Phenotype

*mix-1* null mutants *(mn29)* and *(mn182)* arrest as L1 or L2 larvae and can live for several days in this state. Worms examined by Nomarski microscopy show pleiotropic phenotypes consistent with defects in the timing and completion of cell division. Defects commonly observed include: aberrant nucleolar shape; abnormal nuclear morphology; unidentified cells and cell divisions at inappropriate times and locations; failure of V-cell divisions in L2; asymmetric gonad primordium; undefined, thick, abnormal, or fused L1 alae; disorganized ventral nerve cord; serpentine intestine; and constipation. Most cell migrations, cell fates, and body plan features are relatively normal in arrested larvae.

### Antibody Preparation

A PCR fragment encoding MIX-1 amino acids 1–384 was cloned into the T7 expression vector pRSET B (Invitrogen), in-frame with DNA encoding six histidines. The recombinant protein was purified by nickel-chelate chromatography and SDS-PAGE. Rabbit antisera were affinity purified against column-bound antigen as in Harlow and Lane (1988). The specificity of MIX-1 antibodies was demonstrated by three criteria: the lack of antibody staining in dead embryos homozygous for *mnDf90*, a large deletion that removes *mix-1* and neighboring genes; complete depletion of the staining activity in the MIX-1 antisera by incubation with MIX-1 fusion peptide; and failure of the preimmune sera to stain wild-type embryos. DPY-27 mouse antibodies were directed against amino acids 1–409 (Chuang et al., 1994). For costaining experiments with rabbit antiphosphorylated histone H3 antibodies, MIX-1 antibodies were directly labeled with FITC as in Harlow and Lane (1988).

### Biochemical Analysis

Nuclei were prepared and immunoprecipitations performed with the appropriate affinity-purified antibody as in Chuang et al. (1996). Nuclear extracts were prepared in parallel from equal amounts of wild-type and mutant nuclei. For each IP experiment, equal amounts of the resulting nuclear extract and immunoprecipitating antibody were used. For Western analysis, IPs from wild-type and mutant extracts were divided into equal parts and the blots probed with equal amounts of detecting antibody. Mouse antibodies were used to probe all blots to reduce background caused by the use of rabbit antibodies in the immunoprecipitation reactions. Development times for lanes probed with the same antibody were identical, except for Figure 1C, lane 2, which was developed longer than lane 1.

The stability of the DPY-27/MIX-1 interaction was determined by immunoprecipitating DPY-27 with MIX-1 antibodies (Chuang et al., 1996), except for the addition of washes with 0.6, 0.8, or 1 M KCl. No significant loss of DPY-27 protein occurred through the 0.8 M

KCl wash, and ~50% of DPY-27 protein remained bound during the 1 M KCl wash.

### Antibody Staining

Dosage compensation–defective strains used for antibody staining are described in Chuang et al., (1996); Lieb et al. (1996); and Davis and Meyer, (1997). Greater than 1000 embryos of each mutant genotype were scored; all embryos of the same genotype exhibited similar staining. Adults and embryos were prepared and stained as in (Chuang et al., 1994). For double-staining experiments with FITC-conjugated MIX-1 antibodies, UNC-86 antibodies were used as a control to show that no bound secondary antibodies diffused laterally to the FITC-conjugated MIX-1 antibody.

Maternally rescued *mix-1(b285)* animals were picked to Superfrost Plus (Fisher) charged slides and dissected in M9 buffer for analysis of their gonads and progeny. Slides were then placed directly on dry ice, the cover slip removed with a razor, and the slide placed in 95% ethanol for 1 min. The slide-bound embryos were fixed and stained as in Chuang et al. (1994).

### Suppression of *sdc-3*(Tra) by *mix-1(mn29)*

Because 38% of *sdc-3(y52*Tra*) unc-76(e911)* homozygous animals are hermaphrodites at 25°C, and >99% of these animals are masculinized (Tra) at 20°C (DeLong et al., 1993), we obtained homozygous *sdc-3(y52*Tra*) unc-76(e911)* hermaphrodites by growing *sdc-3(y128)/ sdc-3(y52*Tra*) unc-76(e911)* worms at 25°C. The homozygous *sdc-3(y52*Tra*) unc-76(e911)* Unc hermaphrodite progeny were then shifted to 20°C for mating with *unc-4(e120) mix-1(mn29)/mnC1 dpy-10(e128) unc-52(e444)* males. Twenty wild-type progeny, presumably *unc-4(e120) mix-1(mn29)/++*; *sdc-3(y52*Tra*) unc-76(e911)/++* or *mnCl dpy-10(e128) unc-52(e444)/+++*; *sdc-3(y52*Tra*) unc-76(e911)/++*, were cloned to individual plates at 20°C. All classes of progeny were counted and scored from each animal. The control animals carrying the *mnC1* balancer were distinguished from the others by the presence of Dpy Unc animals in the progeny. The ratio of *sdc-3(y52*Tra*) unc-76(e911)* males to hermaphrodites was used to determine the degree to which *mix-1(mn29)/+* could suppress the masculinization of *sdc-3(y52*Tra*)* animals.

### Acknowledgments

Correspondence should be addressed to B. J. M. We thank the *Caenorhabditis* Genetics Center for providing strains; R. K. Herman for permitting the renaming of *let-29*; Y. Kohara for *mix-1* cDNA clones; Y. Wei and C. D. Allis for phosphorylated histone H3 antibodies; H. Bass for tubulin antibodies; M. Sym for Nomarski analysis of *mix-1(mn29)* worms; J. Waddle for physical map information; and T. Cline, D. Pasqualone, D. Reiner, and J. Rine for comments on the manuscript. This work was supported by U. S. P. H. S. Grant R37 GM30702 and American Cancer Society Grant DB-5B (to B. J. M.) and U. S. P. H. S. Grant T32 GM07127 (to J. D. L.). B. J. M. is an investigator of the Howard Hughes Medical Institute.

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### GenBank Accession Number

The accession number for *mix-1* is U96387.